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4. Jarvis et al
   Infection and Immunity 64(11):4826-4829, 1996

5. Kenny et al
   Proc. Natl. Acad. Sci. 92(17):7991-7995, 1995

Thank you

Enteropathogenic *E. coli* Exploitation of Host Epithelial Cells[a]

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ENTEROPATHIC E. COLI-MEDIATED DISEASE

Escherichia coli is an extremely versatile pathogen. In addition to being a member of the normal intestinal flora, *E. coli* also causes bladder infections, meningitis, and diarrhea. Diarrheogenic *E. coli* contain at least five types of *E. coli*, which cause various symptoms ranging from cholera-like ones to extreme colitis.[1] Each type of diarrheogenic *E. coli* possesses a particular set of virulence factors, including specific adhesins, invasins, and/or toxins, which are responsible for causing a specific type of diarrhea. One of these groups, enteropathogenic *E. coli* (EPEC), is a predominant cause of infant diarrhea worldwide. In addition to isolated outbreaks in day-care centers and nurseries in developed countries, EPEC poses a major endemic health threat to young children (< 6 months) in developing countries, where it has a high mortality rate.[2] Worldwide, EPEC is the leading cause of bacteria-mediated diarrhea in children, and it is estimated to kill up to one million children each year. EPEC disease is characterized by watery diarrhea of varying severity, while vomiting and fever often accompany fluid loss.

Despite the significance of EPEC-mediated disease, little is known about how this pathogen actually causes disease. Unlike other *E. coli* diarrheas such as enterotoxigenic *E. coli*, EPEC diarrhea is not mediated by a toxin. Instead, EPEC binds to intestinal surfaces of the small bowel. A characteristic histologic lesion, called the attaching and effacing (A/E) lesion, occurs.[3] A/E lesions are marked by dissolution of the intestinal brush border surface and loss of epithelial microvilli (effacement) at the sites of bacterial attachment. Once bound, EPEC reside upon a

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MEDIATED DISEASE

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cup-like projection or pedestal upon which adherent bacteria reside. Underlying this pedestal in the epithelial cell are several cytoskeletal components, including actin, alpha-actinin, ezrin, talin, and myosin light chain.[4,5] Formation of the A/E lesion appears to be responsible for fluid secretion and diarrhea; however, mechanistically this remains to be proven. It has been suggested that disruption of the brush border and microvilli may be responsible for diarrhea. Although EPEC can enter (invade) tissue culture cells,[6] it does not normally cause invasive disease and rarely penetrates the intestinal barrier.

EPEC belongs to a group of pathogenic organisms that form A/E lesions, including enterohemorrhagic *E. coli* (EHEC), several EPEC-like animal pathogens that cause disease in rabbits (RDEC), dogs, pigs (PEPEC), and the like, and some isolates of *Citrobacter freundii*, *Hafnia alvei*, and probably *Helicobacter pylori*. These organisms all cause cytoskeletal rearrangement and pedestal formation on relevant host epithelial cells. EHEC, which causes enteric colitis (hamburger disease), can also cause hemolytic uremic syndrome in approximately 10% of cases. EHEC

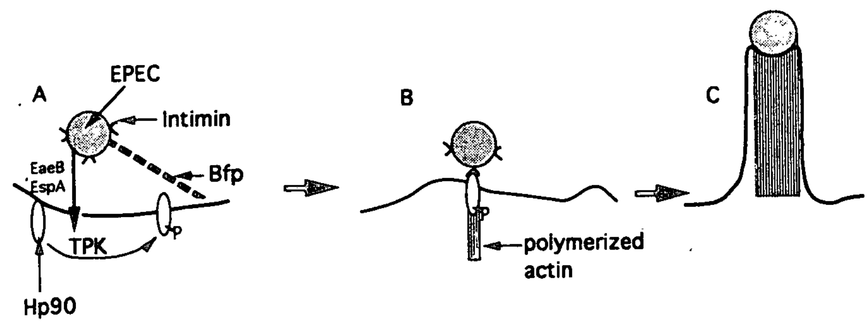

FIGURE 1. Model of the various stages of enteropathogenic *E. coli* (EPEC) interaction with epithelial cells. (A) EPEC initially binds to epithelial cells via its bundle-forming pilus. At least two EPEC-secreted proteins (EaeB and EspA) cause activation of host signal transduction pathways, including a tyrosine protein kinase (TPK) which phosphorylates a host membrane protein (Hp90). (B) Once Hp90 is phosphorylated, EPEC intimin then binds to it, and polymerized actin and related cytoskeletal proteins accumulate beneath the adherent bacteria. (C) Actin polymerization continues beneath adherent bacteria, developing into a fully developed attaching/effacing lesion.

appears to possess all of the EPEC virulence factors needed for A/E lesion formation, but it has an additional shiga-like toxin that contributes to its increased pathogenesis.

MECHANISMS OF PATHOGENICITY

Initial Adherence. Recently, significant progress has been made in defining the bacterial and host factors involved in formation of attaching and effacing lesions. (See FIG. 1 for an outline.) Initial bacterial adherence is dependent on the presence of a 55–70 MD plasmid that is common to EPEC strains. This process is mediated by a plasmid-encoded bundle-forming pilus (BFP) and possibly other factors.[7] Mutants in EPEC that are defective in initial adherence produce fewer A/E lesions on epithelial cells, but these lesions are indistinguishable from those caused by parental EPEC.

Signal Transduction. When EPEC interact with cultured epithelial cells, several signal transduction pathways are activated in the epithelial cells, including the release of the eukaryotic secondary messengers, IP_3, and intracellular calcium.[8,9] EPEC binding to cultured epithelial cells also causes tyrosine phosphorylation of a host 90-kD membrane protein, Hp90, which is not normally phosphorylated in uninfected cultured cells.[10] The addition of tyrosine kinase inhibitors inhibits the phosphorylation of Hp90 and EPEC uptake into epithelial cells. Hp90 phosphorylation appears to precede IP_3 fluxes and cytoskeletal rearrangements.[8]

All of the EPEC genes known to be involved in A/E formation (except the plasmid-encoded regulator *per*) are found within a unique contiguous region in the EPEC chromosome.[3] Several bacterial loci have been identified that are involved in activating epithelial signal transduction. Strains containing mutations in *eaeB,* a gene found downstream of the intimin gene *eaeA* (see below), do not stimulate signal transduction or cytoskeletal rearrangement.[11] Strains cured of the EPEC virulence plasmid are still capable of activating epithelial signal transduction pathways and organizing the underlying cytoskeletal structure; however, their efficiency at these events is significantly decreased, presumably because of the loss of the plasmid-encoded bundle-forming pilus and a plasmid-encoded positive regulator. In addition to *eaeB,* Tn*phoA* mutants belonging to Class 4 (*cfm* mutants) are also unable to stimulate signal transduction.[10] We recently found that another locus upstream of *eaeB,* called *espA* (*E. coli*-secreted *protein* A), is also needed to stimulate epithelial signals.[12]

We recently showed that when EPEC is grown in tissue culture media, five bacterial proteins (110, 40, 39, 37, and 25 kD) are secreted into the supernatant medium.[13] Amino terminal sequencing identified the 37 kD as EaeB (a protein needed to trigger signal transduction[11]), and the 25 kD protein matched the predicted product of *espA,* EspA.[12] The 39-kD protein is homologous to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with 14 of 16 amino acids at its amino terminus being identical to GAPDH. *cfm* mutants were unable to secrete any of these proteins except the 110-kD protein.[13] We recently cloned and sequenced the gene encoding the 110-kD protein (Stein and Finlay, manuscript in preparation). This protein is homologous to a hemagglutinin found in an avian pathogen *E. coli* and uses an IgA protease secretion mechanism. However, by constructing internal gene deletions, we found that the 110-kD protein is not needed for signal transduction and is not secreted by several A/E-causing organisms such as RDEC, *C. freundii,* and *H. alvei* (Stein and Finlay, manuscript in preparation). It also appears that the 39- and 40-kD proteins are not encoded within the 35-kb locus of the enterocyte effacement region, based on DNA hybridization studies and DNA sequence analysis. This region encodes all the factors necessary for A/E formation when placed in HB101 (J. Kaper, personal communication), and thus these two proteins are probably not needed for A/E lesion formation. Characterization of the *cfm* insertions led to the identification of a "Type III" secretion system in EPEC, encoded by the *sep* genes.[13,14] Such secretion systems are used by several bacterial pathogens to export virulence factors out of the bacteria and into contact with mammalian cells. Examples include the invasion systems of *Shigella* and *Salmonella* species, the *Yersinia*-secreted proteins including a tyrosine phosphatase that enters phagocytic cells, and harpins from plant pathogens.[15] Collectively, this information indicates that EPEC secretes at least two molecules (EaeB and EspA) that are critical for activating signal transduction and cytoskeletal rearrangement in epithelial cells, and EPEC has a specialized secretion system for exporting these molecules.

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Intimate Adherence. Intimin is the product of a bacterial chromosomal locus, *eaeA*, and is a 94-kD EPEC outer membrane protein that is needed for intimate adherence.[16] We found that *eaeA* mutants form immature A/E lesions and do not organize phosphotyrosine proteins and cytoskeletal components beneath adherent bacteria, although epithelial signal transduction is still activated.[10] Intimin appears to participate in reorganization of the underlying host cytoskeleton after other bacterial factors stimulate epithelial signal transduction.[10] Although cloned intimin in nonpathogenic *E. coli* does not mediate adherence,[16] we recently showed that if an *eaeA* defective strain of EPEC is added to epithelial cells (to stimulate signal transduction) before the addition of the cloned intimin expressed in *E. coli* HB101, bacteria expressing the cloned intimin now adhere to epithelial cells.[17] If EPEC mutants that are defective for stimulating signal transduction (such as *eaeB* or *cfm*) are used to preinfect monolayers, organisms containing the cloned intimin do not adhere. This indicates that EPEC-induced signal transduction pathways are needed before successful intimin-mediated adherence to epithelial cells.

Further support for this hypothesis comes from studies using a purified fusion peptide consisting of 280 amino acids of the carboxyl terminus of intimin fused to the maltose-binding protein (MBP).[17,18] We found that this fusion peptide (MBP/Int) adheres to epithelial cells *only* when the monolayer is previously infected with EPEC (or the *eaeA* mutant) to preinduce signals.[17] If these signals are blocked with a tyrosine kinase inhibitor (which blocks signaling), binding of the fusion peptide to epithelial cells is also blocked. Not surprisingly, peptide binding is not affected by treatment of the monolayer with cytochalasin D, indicating that actin rearrangement is not needed for intimin-mediated binding.

Hp90 is an epithelial membrane-localized protein that localizes beneath adherent organisms at the tip of extended pseudopods.[17] This protein interacts with intimin and is a candidate for the intimin receptor. If EPEC is added to epithelial cells and then removed by detergent extraction, Hp90 remains associated with the bacteria; however, if an *eaeA* mutant is used, Hp90 is not isolated with the bacteria.[17] Hp90 is also coimmunoprecipitated with the intimin-maltose binding protein fusion peptide, but only if prior signals are induced in the epithelial cells by EPEC strains.

Cytoskeletal Rearrangement and Pedestal Formation. As just described, the A/E lesion (or pedestal) formed by EPEC on association with epithelial cells is associated with the assembly of highly organized cytoskeletal structures in the epithelial cells immediately beneath adherent bacteria. Although this pedestal usually raises the bacterium slightly above the epithelial cell surface, we recently showed that EPEC can trigger extended pseudopod formation, with projections extending up to 10 μ above the epithelial cell surface with the bacteria located extracellularly at the tip of these extensions.[17] The stalk of these extended pseudopods contains polymerized actin, whereas Hp90 is localized only at the tip of these structures beneath EPEC. Extended pedestals are not seen when strains containing mutations in *eaeA*, *eaeB*, or *cfm* are used, reinforcing the linkage between signal transduction events and cytoskeletal rearrangement.

The product of the *eaeA* gene, intimin, appears to be critical for organizing cytoskeletal rearrangements. *eaeA* mutants trigger signals in epithelial cells and cause generalized actin accumulation near adherent organisms, but they are unable to organize the cytoskeleton into defined structures that lead to pedestal and extended pseudopod formation. Additionally, they do not invade epithelial cells, even if complemented with signal transduction-defective EPEC mutants. Further support for the role of intimin comes from experiments performed with the cloned EPEC intimin expressed in nonpathogenic *E. coli* HB101. If EPEC containing a defective *eaeA* gene are first added to epithelial cells followed by strains containing

cloned intimin, only HB101 harboring intimin, but not the *eaeA* mutant, organize the cytoskeleton into pedestals and extended pseudopods.[17] This indicates that intimin molecules direct the final condensation and organization of the host cytoskeleton from their outer membrane location on the adherent *E. coli.* In addition to mediating intimin binding, Hp90 also probably plays a significant role in organizing the host cytoskeleton. It may even serve as a bridge, linking intimin in the bacterial outer membrane to the host cytoskeleton on the other side of the epithelial cell membrane.

Production of Diarrhea? Despite our increasing knowledge of the bacterial factors and host molecules that mediate EPEC interactions with epithelial cells, the actual molecular mechanisms that cause diarrhea remain undefined. EPEC strains lacking intimin are significantly decreased in their ability to cause diarrhea in human volunteers.[19] One or more of the events associated with the formation of A/E lesions also cause diarrhea. However, signal transduction mutants such as *eaeB* or *espA* have not been tested for virulence in humans or relevant animal models. In addition to the morphologic rearrangements that occur on the apical surface of epithelial cells, EPEC also causes a large decrease in transepithelial resistance in polarized Caco-2 epithelial cell monolayers.[20] This disruption does not appear to be due to alterations in tight junctions, but instead it affects a transcellular pathway. Mutants defective for signal transduction and *eaeA* mutants do not cause this loss in transepithelial resistance, indicating that this process is linked to these events. It is possible that such transepithelial disruptions occur *in vivo,* which would lead to ionic imbalances and possibly diarrhea.

By using whole cell patch clamping technology, we recently found that EPEC causes significant depolarization of individual HeLa cells (Stein, Mathers, and Finlay, in preparation). Although *eaeA* mutants still caused depolarization, both *eaeB* and *cfm* mutants did not depolarize cells, indicating that EPEC-secreted proteins that affect epithelial signaling are needed for these events. The occurrence of such a process in the gut would reduce the electrochemical gradient available for sodium ion absorption from the gut lumen, thereby contributing to ionic imbalance, fluid loss, and diarrhea.

SUMMARY

Enteropathogenic *E. coli* (EPEC) is a leading cause of neonatal diarrhea worldwide. These organisms adhere to the intestinal cell surface, causing rearrangement in the epithelial cell surface and underlying cytoskeleton, resulting in a structure termed an attaching/effacing (A/E) lesion. A/E lesion formation is thought necessary for EPEC-mediated disease. EPEC secretes several proteins that trigger signal transduction, intimate adherence, and cytoskeletal rearrangements in epithelial cells. Additionally, it produces intimin, an outer membrane product that mediates intimate adherence. Together these various bacterial molecules contribute to the intimate relationship that is formed by EPEC with host epithelial cells which results in A/E lesion formation and diarrhea.

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